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## (54) Title: CELL SORTING TECHNIQUE AND APPLICATIONS THEREOF

## (57) Abstract

Methods and compositions are described for obtaining hybrid cells by fluorescence activated cell sorting wherein two cell populations are each labeled with different fluorescent dyes having similar excitation wavelengths but distinct emission wavelengths, one member of the dye panel having the property of being transported rapidly from cells and the methods illustrated herein present the transport process thereby enabling hybrid cells to be identified and isolated that are labeled with both fluorescent dyes.

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## CELL SORTING TECHNIQUE AND APPLICATIONS THEREOF

This invention is in the field of cell biology, and demonstrates a cell sorting technique whereby hybrid cells are identified and isolated. More specifically, a method is shown whereby hybrid cells, preferably antibody secreting cells, are 5 selected using a fluorescence activated cell sorter.

Techniques which permit the formation of hybrid cells have significantly facilitated an understanding of various biological phenomena, as well as having had profound practical applications, most notably the formation of antibody secreting hybrid cell-lines. Kohler, G. et al, Nature 256:495 (1975). Basically, what is 10 involved in forming hybrid cells is fusing one or more target cells with an appropriate fusogen, and subsequently selecting out of the mixture the hybrid cells. Methods for fusing cells, as well as methods for selecting hybrids are known in the art.

The two most widely employed fusogens, or cell fusing chemicals, are 15 polyethylene glycol (PEG) or inactivated virus, particularly Sendai virus. Ringertz, N. and Salvage, R. Cell Hybrids Chapters 4 (p. 29) and 5 (p. 46), Academic Press (1976). Because of the ready availability of PEG, and because Sendai virus requires time-consuming production, titration and inactivation steps, PEG is by far the favored fusogen. PEG is available in various molecular weight formulations, and particular 20 formulations are preferred fusogens. Gefter, M. et al., Somat. Cell Genet. 2:231, (1977). Hybrid cells are generally selected using one of two methods. The first consists of forming hybrids from cells that have drug resistant genetic markers that facilitate growth of the hybrids in defined media, but not the non-fused cells. The second consists of introducing two distinct fluorescent markers into each of the cells 25 that make up the hybrids, and subsequently, selecting hybrids that exhibit both fluorescent molecules using a fluorescent activated cell sorter machine.

Fluorescent activated cell-sorting techniques have several advantages over isolating hybrids using classical biochemical selection techniques. First, and most apparent is that there is no need to produce cell lines having the genetic markers that 30 permit selection in drug supplemented media. This is often a very time consuming and arduous task. Second, after the fusion procedure is carried out, a biochemical selection procedure is not required wherein the hybrids and non-fused cells are grown

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in drug selection medium in order to select against the non-fused cells. Third, the efficiency of a particular fusion technique can be determined immediately using fluorescent activated cell sorter techniques. In contrast, if the cells are selected using drug markers it is necessary to wait a significant period of time for the cultures to 5 grow up, and be depleted of non-fused cells.

Despite the advantages of selecting hybrid cells using fluorescent activated cell sorting techniques, the method is not generally applicable to a wide variety of cells because of the difficulties associated with identifying compatible combinations of dyes. This is particularly true if mouse hybrids are selected because of the paucity 10 of fluorescent dyes that are taken up by the cells, and additionally retained therein for a time sufficient to carry out the selection process. This is particularly true of mouse antibody secreting cells, either hybridomas, triomas, or quadromas. In part, this is because mouse cells are known to have membrane transport systems that can remove from the cell fluorescent dyes that are useful in fluorescent activated cell 15 sorting.

Another drawback associated with fluorescent activated cell sorting techniques is that, to date, there have not been identified pairs of fluorescent dyes that can distinguish between dead and viable cells. Thus, current methods select for hybrids that consist of dead cells as well as viable cells.

20 Accordingly, an object of the instant invention is the presentation of fluorescent dye compositions, and methods of using the same that permit the selection of hybrid cells.

Another object of the invention is the illustration of fluorescent dye composition and methods of using the same that permit the selection of hybrid cells, 25 preferentially rodent hybrids, and more preferentially antibody secreting rodent hybrids.

A further object of the invention is the identification of a pair of fluorescent dyes, and methods of using the same, that are taken up intracellularly by viable cells thereby enhancing the selection of viable hybrids wherein the hybrids are formed 30 from a fusion of cells each containing one member of the dye pair.

An additional object of the invention is the identification of a pair of fluorescent dyes, and methods of using the same, to preferentially select viable hybridoma rodent cells wherein one member of the dye pair has the property of being

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transported rapidly from the cells, and the methods illustrated herein prevent the transport process, thereby enabling utilization of the dye to select rodent hybrids.

Figure 1 shows the growth curve of 520C9 cells labeled with either rhodamine 123 at 0.5ug/ml, or hydroethidine at 10ug/ml. Also shown is the growth 5 curve of control cells not labeled with either dye.

Figure 2 shows the growth curve of 3G8 cells labeled with rhodamine 123 at 0.5ug/ml, or hydroethidine at 10ug/ml. Also shown is the growth curve of control cells not labeled with either dye.

Figure 3 shows the FACS profile of 520C9 cells labeled with rhodamine 10 123.

Figure 4 shows the FACS profile of 3G8 cells labeled with hydroethidine.

Figure 5 shows the FACS profile of a mixture of unfused 520C9 and 3G8 cells.

Figure 6 shows the FACS profile of a mixture of cells containing fused 15 520C9 and 3G8 cells.

Figure 7 presents the chromomycin A3 DNA staining profile of 520C9 and 3G8 cells, and 3E11, a hybrid hybridoma derived from the fusion of these cell lines.

Figure 8 shows the chromomycin A3 DNA staining profile of 520C9 and 3G8 cells and 4H3, a hybrid hybridoma derived from the fusion of these cell lines.

20 Figure 9 shows the cytotoxic effect of 5A5, and 4H3 culture supernatants towards labeled SKBr3 cells.

Figure 10 shows the cytotoxic effects of 5A5, 4H3, and corresponding subclones towards tritium labeled SKBr3 cells.

Figure 11 illustrates the OD<sub>280</sub> Sephadryl 200 elution profile of ascites fluid 25 containing antibody secreted by the hybrid hybridoma, 2B1.

Figure 12 shows the OD<sub>280</sub> DEAE Sepharose chromatography elution profile of antibody fractions of 2B1.

Figure 13 shows the SDS PAGE gel electrophoretic pattern of the DEAE fractions 52-62, 78-85 and 102-110.

30 Figure 14 shows the cytotoxic activity of DEAE purified 2B1 against chromium labeled SKBr3 cells.

The invention shown herein describes a method for selecting hybrid cells using fluorescent activated cell sorting techniques. Generally this involves selecting a

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pair of fluorescent dyes that are not toxic at the concentrations employed, and that are taken up intracellularly and retained therein for a time sufficient to perform the cell sorting procedure. Additionally, the fluorescent dyes will have the further property of exhibiting close excitation wavelengths but distinct emission wavelengths.

5 The cells are fused using a suitable fusogen, and selected preferably using a fluorescent activated cell sorting machine.

#### Labeling of Cells with Fluorescent Dyes

A variety of hybrid cell types can be produced by the methods of this invention. The invention is particularly useful, however, to obtain cell lines that 10 secrete bifunctional monoclonal antibodies. A bifunctional antibody exhibits two distinct antigen binding sites. Triomas and quadromas are two examples of cell lines that can secrete bifunctional monoclonal antibodies. Triomas are generally formed by fusion of a hybridoma and a lymphocyte, whereas quadromas are generally formed by somatic cell fusion of two hybridomas. The hybridomas and lymphocytes each 15 produce a monospecific antibody, that is, an antibody that exhibits two binding sites for the same antigen. However, triomas and quadromas synthesize light and heavy chains of both parental types, that is, of both the hybridomas or lymphocytes, which combine to produce bispecific antibody.

The initial step in the identification and isolation of hybrid cells by the 20 methods of the invention involve incubating, in a suitable physiological solution, those cells which are sought to be fused in separate tubes containing the fluorescent dyes of choice. The cells are allowed to incubate until an amount of fluorescent dye is taken up which is sufficient to be subsequently detected in a hybrid.

A wide variety of physiological solutions can be used to incubate cells in the 25 presence of the fluorescent dyes. For instance, the cells can be incubated for short periods of time in physiological solutions lacking nutrients, such as phosphate buffered saline, or if a particular fluorescent dye requires longer incubation times to effect uptake of detectable amounts of dye, then the cell line is more appropriately incubated in a solution supplemented with nutrients, preferably cell culture media, to 30 prevent cellular deterioration. The pH of the solution is expected to be in the range of about 7.4; however, variations are anticipated to be usable that do not significantly affect the uptake of the fluorescent dyes, nor adversely affect the viability of the

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cells.

The effect of fluorescent dyes on cell viability can be measured using techniques well known to those skilled in the art. A most useful technique is described by Mosmann, T., J. of Immunol. Methods, 65:55 (1983) which measures 5 cell viability as a function of cell number. Thus, the effect of fluorescent dyes on both cell viability and cell number can be measured simultaneously. The assay is based on the conversion of colorless tetrazolium salts by mitochondrial dehydrogenase enzymes to a detectable colored product. A favored tetrazolium salt is MTT, or (3-(4,5- dimethylthylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide).

10 The fluorescent dyes that are usable in the instant invention have several important characteristics. First, as mentioned above, they should not be toxic to cells at the concentrations necessary to form and detect viable hybrids using a fluorescent activated cell sorter. Second, the dyes should also emit light at wavelengths that are readily detectable using suitable fluorescence detection devices. Third, once the dyes 15 are taken up by the cells the rate of efflux of the dyes from the cells during the washing and measurement period should not be so great as to reduce the amount of dye inside the cell to a non-detectable level. Virtually any dye pair having these properties will perform adequately in the instant invention. However, most preferred are the dyes rhodamine 123 and hydroethidine. In most cell types these dyes are 20 readily taken up and maintained. However, rodent cells, particularly mouse cells, exhibit a high efflux rate for rhodamine 123. It has been determined, however, that if cells that are incubated with rhodamine 123 are also incubated with a calcium channel blocker, that is, a molecule that interferes with the transport of calcium across the plasma membrane, that rhodamine 123 is maintained inside the cells for 25 longer times than if the channel blocker is absent. Without wishing to be held to any particular theory, it is thought that rhodamine 123 is exported from the cell by a mechanism that is linked to calcium ion transport, and that by interfering with calcium ion transport using a suitable calcium channel blocker that efflux of rhodamine 123 is also blocked, and the dye is hence maintained inside the cell.

30 Thus, in those instances where rhodamine 123 is one member of the dye pair, and the cells that are sought to be fused are rodent cells, in order to maintain a sufficient concentration of rhodamine 123 intracellularly, a calcium channel blocker should be present in the solution during the time that the cells are labeled with the dye, and

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5 during the subsequent washing, cell fusing, and fluorescence activated cell sorting procedures. While a variety of calcium channel blockers are known in the art, the preferred calcium channel blocker of the instant invention is verapamil. It is commercially available from Sigma Chemical Corporation. The effective concentration of verapamil can be determined empirically using techniques known in the art.

10 As alluded to above, the concentrations of the fluorescent dyes in the labeling solution must be non-toxic to the cells, yet high enough for the cells to take up an amount that can be detected in hybrids using the fluorescent activated cell sorter after the cells are fused and hybrids formed. For most dyes the concentration employed will be in the  $\mu\text{g}/\text{ml}$  range, and preferably in the range of 0.1 - 20  $\mu\text{g}/\text{ml}$ . Hydroethidine and rhodamine 123 are preferably used at concentrations of about 0.1 - 10  $\mu\text{g}/\text{ml}$  and 1 - 15  $\mu\text{g}/\text{ml}$ , respectively. The most preferred concentrations of these dyes are 0.25 - 0.5  $\mu\text{g}/\text{ml}$ , and 5 - 10  $\mu\text{g}/\text{ml}$  for rhodamine 123 and hydroethidine respectively.

15 Other details regarding labeling of cells with suitable fluorescent dyes are the optimal time that the cells should be incubated with the dyes, as well as the incubation temperature. As mentioned above, the labeling period can vary considerably depending on the cell types used, as well as the concentration of the fluorescent dyes. However, the preferred incubation period is about 10 - 50 minutes, more preferred is 15 - 30 minutes, most preferred is 20 minutes. The cells can be incubated at a variety of temperatures, which will in turn affect the incubation period. The optimal time for the various parameters can be determined empirically. However, the preferred incubation temperature for cells incubated 20 minutes is about 20 37° C, for rhodamine 123 and 20 minutes at room temperature for hydroethidine. After the cell lines are labeled with the appropriate fluorescent dye, they are washed to remove residual dye and incubated in a physiological solution, preferably cell culture media, in preparation for carrying out the cell fusion procedures described below.

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Cell Fusion

The cell lines containing the appropriate fluorescent dyes are combined in a physiologically acceptable solution, and fused using standard cell fusion materials and methods. It is important to note that regardless of the procedure used, in those instances where the cell lines employed exhibit a significant rate of efflux of, for example, rhodamine 123 from rodent cells, that the fusing mixture will contain a suitable calcium channel blocker, preferably verapamil. Fusion can be effected using a variety of fusogens, the preferred fusogen, however, is polyethylene glycol. More preferred are polyethylene glycols having molecular weights in the range of 1500 to 10 4000. The two cell lines can be combined at different concentrations, however, it is preferred that about  $10^6$ - $10^7$  cells of each cell line be utilized.

More specifically, the fusion procedure consists of combining about  $10^6$ - $10^7$  cells of each cell line in a suitable cell culture media, with or without verapamil depending on the type of cell lines fused. The cell mixture is centrifuged to pack the 15 cells, and the cells fused using polyethyleneglycol 1500. The technique employed is described by Kohler and Milstein, *Nature*, 1975, 256:495. Briefly, one procedure whereby cell hybridization can be achieved is by the addition of 0.5 ml of a 50% (v/v) solution of polyethyleneglycol 1500 by dropwise addition over 30 seconds at room temperature, followed by a 30 second incubation at 37°C. The cell suspension 20 is slowly mixed with the addition of 20 ml of cell culture media containing 10% fetal calf serum. Next, cells are gently resuspended in cell culture medium containing 10% fetal calf serum and incubated for 2 -4 hours at 37°C prior to sorting the cells using a fluorescent activated cell sorter machine equipped with an argon laser that emits light at 488 or 514 nm. These wavelengths are particularly useful when using 25 the dye pair hydroethidine and rhodamine 123. Hydroethidine intracellularly is converted to ethidium when excited at either 488 or 514 nm emits a red fluorescence. In contrast, rhodamine 123 when excited at either wavelength emits a yellow fluorescence. Thus, hybrid cells are expected to emit both red and yellow fluorescence.

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Cell Sorting

Hybrids present in the mixture of fused cells can be isolated using a fluorescent activated cell sorter machine. While a variety of such machines are available, we have found that a Coulter EPICS V, or a FACS III Cell Sorter 5 produced by Beckon-Dickinson, Sunnyvale, California, perform adequately. Both are equipped with argon ion lasers. The laser is preferable used at a power setting of about 150 mW, and as mentioned above, at an excitation wavelength of 488, or 514 nm. Standard mirrors and filters are employed to be compatible with, and to collect the fluorescence emitted by, a select pair of fluorescent dyes. Similarly, standard 10 techniques are used to set the cell sort windows that are used to select the hybrid cells, and are described by L. Karawajew, B. Micheel, O. Behrsing and M. Gaestel, J. Immunol. Methods, 1987, 96:265-270.

Identification of Antibody Secreting Hybridomas

As mentioned above, the instant technique is particularly useful to isolate 15 antibody secreting hybrid cells, preferably hybridomas, triomas, and hybrid hybridomas, or as they are more popularly known, quadromas. However, because not all hybrid cells isolated by fluorescence activated cell sorting will secrete antibody, those that do must be identified. As applied to detecting hybridomas, the most general approach involves assaying medium for antibody secreted by hybrid cells 20 using a solid phase assay, although other techniques are known can be used. Similar techniques are available to detect membrane, or non-soluble antigens. In both instances, antigen is bound to a solid support, the support treated with a suitable blocking agent, and antigen detected either directly with labeled hybridoma antibody, or indirectly with a labeled molecule that binds to hybridoma antibody. The latter 25 can be antibody, protein A, or other suitable binding agents. Such assays are known in the art, and are shown by Langone, J. and Van Vinakis, H., Methods of Enzymology, 92, Part E (1983).

Antibody secreted by triomas or quadromas can be identified by the methods described above, but additional assays may be performed to ascertain the bifunctional 30 characteristics and properties of the antibody. As discussed above, antibody produced by triomas or quadromas is bispecific, that is, bifunctional in the sense of having binding affinities for two different antigens within a single antibody molecule.

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Antigen binding may be simultaneous or sequential. In order to confirm that triomas or quadromas can be isolated by the instant techniques antibody secreted by these cell lines can be characterized by any functional test which depends upon the binding of two different antigens by the same antibody molecule.

5 For example, bispecific antibody can be identified using bifunctional antigen screening assays to establish that the antibody does indeed recognize two distinct antigens, and thus is composed of two different antigen combining sites. Such assays are also known in the art, several of which are described in U.S. Patent Nos. 4,634,664; 4,714,681; and 4,474,893. These patents are hereby incorporated by  
10 reference. It is worth noting that U.S. Patent No. 4,634,664 describes triomas, whereas U.S. Patent Nos. 4,714,681 and 4,474,893 presents methods for making and identifying quadromas.

Cellular cytotoxicity assays can also be employed to identify antibody secreted by triomas or quadromas. Cellular cytotoxicity is thought to rely on cell 15 surface receptors on cytotoxic cells, such as monocytes, macrophages, natural killers etc. These receptors are thought to be specific for, and to interact with membrane components on a target cell, thereby causing cell lysis by forming conjugates between the cytotoxic cell and the target cell. If the cytotoxic cell is positioned up against the target cell, cytotoxicity is enhanced. Bifunctional antibody can promote this 20 process by binding to a target cell through one of its combining sites, and to the lysis promoting receptor on the cytotoxic cell through the second combining site, thereby joining the two cell types and causing the cytotoxic cell to deliver a kill signal. The materials and methods for performing these assays are generally known to those who work in this field. Representative assays are described by Mishell and Shiigi, in 25 "Selected Methods in Cellular Immunology", p.130, eds. C. Henry and R. Mishell, publisher W.H. Freeman and Co., San Francisco (1984), and later Herlyn D., Herlyn M., Steplewski, Z., and Koprowski H., "Monoclonal Anti-Human Tumor Antibodies of Six Isotypes in Cytotoxic Reactions with Human and Murine Effector Cells", Cellular Immunol, 1985, 92:105-114. The former reference describes a <sup>51</sup>Cr release 30 assay, whereas the reference shows a <sup>3</sup>H release assay involving measuring the release of tritiated thymidine from lysed cells.

After triomas or quadromas are identified using functional assays as referred to above, the bispecific nature of the antibody can be confirmed by determining the

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composition of isolated antibody preparations. Bispecific antibody should be composed of light and heavy chains of both fusing partners. Antibody can be purified from culture media or body fluids, as the case may be, by conventional immunoglobulin purification procedures such as ammonium sulfate precipitation, gel 5 electrophoresis, dialysis, chromatography, and ultrafiltration, if desired. Ion exchange, size exclusion, or hydrophobic chromatography can be employed, either alone or in combination. Light and heavy chain analysis can be carried out using gel electrophoretic techniques, as well as other techniques known in the art.

Having generally described the invention, examples illustrative of its 10 application will now be presented. However, it will be understood by those skilled in the art that the examples are not intended to be restrictive in any way of the invention.

#### Example I

##### Fluorescent Dye Cytotoxicity Assay

15 Prior to forming hybrid hybridomas or quadromas of the cell lines 3G8 and 520C9, the cytotoxic effect of rhodamine 123 and hydroethidine was determined. 3G8 is a murine hybridoma that secretes monoclonal antibody that recognizes human Fc receptor III present on various cell types, including monocytes, natural killer cells, and macrophages. It is described by Unkeless et al., Annual Review of 20 Immunology, 1988, 6:251. 520C9 is a murine monoclonal antibody that recognizes an antigen present on breast cancer cells. It is described in U.S. patent 4,753,894. The foregoing articles are hereby incorporated by reference in their entirety.

Cell growth and viability was determined using the MTT assay as described by Mosmann, T., above. In order to establish that the MTT assay was reflective of 25 cell number, experiments were carried out to insure that the assay exhibited linearity over the appropriate range of cells. Each of the above cell lines was tested, and the results are shown in Figures 1, and 2 for 520C9 and 3G8 respectively. For both cell lines the assay was linear at least up to  $10^4$  cells/well.

Next, the same cell lines were labeled in the presence or absence of either 30 rhodamine 123 or hydroethidine, and subsequently cultured for three days during which cell number was measured. Cells were labeled in the presence of 0.5  $\mu$ g/ml rhodamine 123 plus 10  $\mu$ M verapamil, or 5 or 10  $\mu$ g/ml hydroethidine. Between

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10,000-20,000 cells/well were labeled with hydroethidine at room temperature, or rhodamine 123 at 37°C at room temperature for 20 minutes, washed twice and then cultured in dye free medium. Figure 1 reveals that neither rhodamine 123 plus verapamil, nor hydroethidine (10 µg/ml) inhibit the growth of 520C9 cells. The 5 growth rate and cell numbers are virtually identical when compared to cells grown in medium lacking these compounds.

Figure 2 shows the results for the cell line 3G8. Neither rhodamine 123 plus verapamil, nor hydroethidine at 5 µg/ml are inhibitory. However, hydroethidine at 10 µg/ml does inhibit 3G8 growth starting at about 24 hours.

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### Example II

#### Labeling of Cells with Fluorescent Dyes

The procedure for labeling cells with fluorescent dyes consisted of incubating about  $2 \times 10^7$  cells of 3G8, or 520C9 with either hydroethidine, 10 µg/ml, or rhodamine 123, 0.5 µg/ml. Cells incubated in rhodamine 123 also received 10 µM 15 verapamil. Hydroethidine labeling occurred at room temperature and in the dark for 20 minutes. Rhodamine 123 labeling occurred at 37°C for 15 minutes, also in the dark. Following the incubation periods, the cell lines were washed twice with serum-free Iscove's medium containing 10 µM verapamil, and fused as described in Example III.

20

### Example III

#### Formation/Isolation of Hybrid Hybridomas

Hybrid hybridomas were formed by fusing 3G8 cells to 520C9 cells. The initial step in the procedure consisted of labeling the cells as described in Example II, and subsequently fusing them with polyethylene glycol. The fusion procedure 25 consisted of combining about  $2 \times 10^7$  cells of each cell line in 50 ml Iscove's medium, and pelleting the cells by centrifugation at 400 rpm for 5 minutes. The supernatant was removed, and the cells washed twice with serum free Iscove's medium containing 10 µM verapamil. This procedure was conducted at room temperature, while subsequent steps were done using solutions at about 37°C.

30 To the cell pellet consisting of 3G8 and 520C9 cells was added 180 µl of Iscove's medium without serum, and 100 µl of this mixture was transferred to a 50

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ml centrifuge tube. This suspension was used to form the hybrids, while the remaining volume served as a control. Fusion was carried out by adding 0.4 ml of a 50% polyethylene glycol solution warmed to 37°C to the cell pellet over a 1 minute period. The mixture was gently swirled for an additional minute to expose as many 5 cells as possible to the fusogen. Next, 0.5 ml of serum-free Iscove's medium was gently added to the mixture over a 1 minute period, and this step repeated once. 7 ml of serum-free Iscove's was added over a 2-3 minute period with continuous stirring, followed by transferring the mixture to a T-150 flask containing 80 ml of Iscove's medium supplemented with 10% fetal calf serum. The mixture was 10 incubated at 37°C in an atmosphere of 95% air, 5% CO<sub>2</sub> for 2-4 hours. Finally, the flask was removed from the incubator, and stood upright for 10 minutes to allow cells to settle to the bottom of the flask. 60 ml of the supernatant was aspirated off, and the remaining mixture sorted to obtain hybrids using a FACS machine as described in Example IV, below.

15

Example IV

Stained cell suspensions were analyzed and sorted using a Coulter EPICS V cell sorter. Rhodamine 123 and hydroethidine were excited at 488 nm with a argon laser at 150 mW power. A 488 nm dichroic mirror was used for collecting right angle light scatter, while a 550 nm dichroic mirror was used in combination with a 20 525 nm band pass filter and a 630 long pass filter to collect the rhodamine 123 and hydroethidine fluorescence, respectively. Forward angle light scatter (FALS), log right angle light scatter (LRALS), log green fluorescence and log red fluorescence were measured simultaneously. Dead cells and clumps were first gated out according to their FALS in combination with their LRALS properties. The second gate was 25 constructed around the double-stained cells. Both gates were used to determine the percentage of double-stained cells (the second gate was gated on the first gate, i.e. FALS vs LRALS).

The double-fluorescent cells were sorted in either a round bottom 96-well microtiter plate, or a flat bottom 12-well plate under sterile conditions using a 30 Coulter autoclone system. Single cells were sorted into 96-well plates, while large numbers of cells were sorted into a single well of a 12 well plate. The sorting gates were constructed on both windows (FALS vs LRALS, LGFL vs LRFL). Since both

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rhodamine 123 and hydroethidine stain only live cells, dead cells can be discriminated from the sort window. Similarly, clumps of live cells containing different fluorescence-stained cells can be gated out according to their light scatter properties.

5 In order to ensure that hybrids would be sorted using the above sorting parameters, the following control was done. 520C9 and 3G8 cells labeled with rhodamine and hydroethidine, respectively, were analyzed and the FACS profiles recorded.

10 Figure 3 shows the dot-plot FACS profile of 520C9 cells labeled with rhodamine 123, while Figure 4 shows the profile of 3G8 cells labeled with hydroethidine. Few if any of either cell type fell into the cell sort window set to select hybrid cells.

15 A second control was done to ensure that only hybrid cells would be sorted using the instant cell sort parameters described above. A mixture of unfused 520C9 and 3G8 cells, that is, the control cells in Example III, was analyzed and the number of cells that fell in the hybrid cell window noted. Figure 5 shows that only about 1.54% of the cells present in the mixture fell within the sort window.

20 In contrast, when a mixture of fused 520C9 and 3G8 cells was sorted, there was a significant increase in the number of cells that fell within the hybrid cell sort window settings. Figure 6 shows, that of the total cells present in the fusion mixture described in Example III, 4.32% fell within the hybrid cell window settings. This indicates that about 2% of the cells were fused.

#### Example V

25 The DNA content of the parent cell lines, and hybrids derived therefrom, was determined in order to confirm that cells selected by the methods of Example IV were indeed hybrid cells resulting from the fusion of 3G8 to 520C9. Standard cell sorting techniques using the DNA stain chromomycin A3 were employed. The techniques are described by Young et al., Proc. Natl. Acad. Sci. USA, 1981, 78(12):7727 and this publication is incorporated by reference. The cells were fixed 30 in 70% methanol and stored at minus 20°C before being assayed. Two independent fusions were done, TS1 and TS3. TS1 produced six clones, while TS3 produced eight clones. Clones 3E11 and 4H3 were from TS1 and TS3, respectively. Figures 7

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and 8, respectively, show that these quadromas exhibit about twice the DNA of the parent cell lines.

Taken together, these data establish that the procedures of the instant invention realize hybrid cells.

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### Example VI

#### Identification of Antibody Secreting Hybrid Hybridomas

Using the procedures described in the preceding examples, hybridomas 5A5 and 4H3, were generated from two independent 3G8 and 520C9 fusions, 5A5 from TS1 and 4H3 from TS3. These hybridomas were shown to secrete bispecific antibody by assaying for bifunctional biological activity initially using a chromium release cytotoxicity assay, which was subsequently confirmed using a thymidine release assay. These experiments were done using 5A5 or 4H3 culture medium containing antibody. Subsequently, 5A5 and 4H3 were subcloned, and the medium from several subclones also tested using the thymidine release assay. The subclones 10 were 2B1 and 2D3 from 5A5; and 3D7 and 3E7 from 4H3. Further, antibody from one of the subclones, 2B1, was purified and tested to demonstrate bispecific activity. A control was also run consisting of medium from a hybridoma 3B5, obtained from the TS1 fusion, that does not secrete bispecific antibody. In addition, the subunit 15 composition of the 2B1 bispecific antibody was analyzed to determine if it exhibited light and heavy chains derived from both 3G8 and 520C9 antibody.

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Chromium labeled SKBr3 cytotoxicity assay: This assay is generally described by Mishell and Shiigi, in "Selected Methods in Cellular Immunology". The cytotoxic cells were prepared as follows. 10 ml of heparinized blood was dispensed into a 50 ml tube and 30 ml of Hank's balanced salt solution containing calcium and 25 magnesium (HBBS Ca-Mg) was added. The tube was inverted several times to mix the solution, and 10 ml of Ficoll-Hypaque slowly pipetted directly to the bottom of the tube. The tube was centrifuged for 25 minutes at 1250 rpm in a IEC DPR-6000 centrifuge at room temperature with the brake off, after which the tube was carefully removed to avoid mixing the solution. Next, the upper layer was removed using a 30 Pasteur pipet down to just above mononuclear layer, which could be distinguished by its cloudy yellow-white appearance. The mononuclear cell layer was collected with a 10 ml pipet, with care not to disturb the red blood cell pellet. This procedure was

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used to process 30 mls of blood. The mononuclear cells so isolated were suspended in a volume of 50 ml with HBSS-Ca-Mg, and centrifuged for 20 minutes at 1250 rpm with the brake in the off position. The supernatant was aspirated to just above the cell pellet, and the cell pellet resuspended. The volume was increased to 50 ml 5 with growth medium, an aliquot removed to determine cell number, and the cells repelleted at 1250 rpm for 10 minutes, again with the brake in the off position. The supernatant was aspirated off, the pellet resuspended and an appropriate amount of growth medium added to adjust the effector/target ratio, or E/T ratio, to 20/1 in a total volume of 0.2 ml/well. This amounted to  $2 \times 10^6$  effector cells/ml.

10 After the effector cells have been isolated and prepared as described above, or simultaneously therewith, the target cells, SKBr3, are prepared. The cells should be labeled with chromium while the cytotoxic cells are being prepared to insure a highly viable cell population. The chromium labeling procedure that was followed is generally described by Mishell and Shiigi, above, with the following changes.

15 SKBR-3 cells were grown to confluence in standard laboratory T150 cm<sup>2</sup> flasks, and fed with fresh medium the day before the experiment. The cell monolayer was washed twice with 10 ml of HBSS-Ca-Mg, and the cells removed from the flask by washing the monolayer with 10ml of versene (EDTA), incubating the flask at 37°C/5%CO<sub>2</sub> for 3-5 minutes, and dislodging the cells by tapping the flask against a solid surface. Next, 10 ml of growth medium was added to resuspend the cells, and the mixture transferred to a 50 ml siliconized polypropylene tube. The volume was 20 adjusted to 50 ml with growth medium, and an aliquot removed to determine the cell number. The cells were pelleted by centrifugation for 10 minutes at 1000 rpm, resuspended in 1ml of growth medium, and 122 uCi of chromium-51 added per confluent flask of SKBr3 cells. The cell suspension was incubated for 45 minutes at 37°C with occasional agitation, after which 14 ml of HBSS-Ca-Mg was added, the cells pelleted, and the supernatant removed. The cells were washed once with 50 ml 25 of HBSS-Ca-Mg and then once in growth medium to remove residual chromium, and resuspended in growth medium at a density of  $2 \times 10^4$  cells/0.1 ml. To standard laboratory 96-well culture plates, 0.1ml of the labeled cells was added, plus 0.1 ml of medium alone or containing the appropriate reagent to be tested, that is, quadroma supernatant, heteroconjugate, or antibody secreted by the parent cell lines. The 30 mixture was incubated at 37°C in an atmosphere of 95% air 5% CO<sub>2</sub> for 45 minutes

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to permit antibody to bind to the effector cells, followed by washing the cells to remove unbound antibody. Next, the supernatant was removed, 0.2 ml of effector cells/well added, and the effector/target cell mixture incubated for 3 hours at 37°C in an atmosphere of 95% air/5%CO<sub>2</sub>. The cell mixture was pelleted, and 0.1 ml of 5 supernatant removed and counted in a gamma counter to determine the per cent lysis and degree of cytotoxicity.

Figure 9 shows that the percent killing associated with antibody secreted by clones 4H3 and 5A5 is significantly greater than that attributed to antibody secreted by either parent cell line, 520C9 and 3G8, that were used to do the fusions that 10 yielded 4H3 or 5A5. Indeed, there is nearly a two fold difference in killing caused by 4H3 and 5A5. The heteroconjugate, 520C9/3G8, showed elevated cytotoxicity similar to that observed for 4H3 and 5A5. Figure 9 also shows that control medium from 3B5 exhibits cytotoxic activity similar to that observed for the medium control. Thus, it is apparent that the quadromas, 4H3 and 5A5, and the subclones derived 15 therefrom, produce antibody that has bispecific activity.

Tritium labeled SKBr3 cytotoxicity assay: Antibody produced by clones 5A5 and 4H3 and their corresponding subclones 2B1, 2D3, and 3D7, 3E7, respectively was assayed for bifunctional cellular cytotoxicity activity as follows. When considering the results, it is important to keep in mind that the antibodies were 20 expected to exhibit binding to Fc receptor cells, as well as to breast cells that display the 520C9 antigen. Human buffy coat cells were pretreated with interleukin-2 and incubated with tritium labeled SKBr3 breast cancer cells in the presence of medium containing antibody, or under various control conditions. SKBr3 cells were labeled for 24 hours by growing the cells in medium supplemented with tritium labeled 25 thymidine (2.5 uCi/ml). After the culture period, the cells were dislodged by trypsin and washed to remove free tritium. The experiment was run in serum free medium, and replicated using buffy coat cells from several human volunteers. Because the antibodies have combining sites that recognize an antigen on the buffy coat cells, as well as on SKBr3 cells, the antibodies should effect enhanced killing compared to 30 their monospecific counterparts. Thus, one control consisted of determining the level of killing due to 520C9 and 3G8 antibodies, alone or in combination, relative to antibody produced by the hybrids. A second control consisted of comparing the level of killing due to a heteroantibody conjugate composed of 520C9 and 3G8. The

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latter was formed using chemical crosslinkers as described by Karpovsky, et al., J. of Experimental Medicine, 1984, 160:1686-1701. Finally, a medium control was also run.

Figure 10 shows that antibody produced by either of the hybrids, 5A5 or 5 4H3, or their subclones, is more effective at killing SKBr3 cells than 520C9 or 3G8 antibody. However, the chemically linked heteroconjugate of 520C9 plus 3G8 is nearly as effective as antibody produced by the clones. Taken together, the results establish that bispecific antibody is produced by 5A5 and 4H3, and their subclones.

Antibody Characterization: Antibody from the 5A5 subclone, 2B1, was 10 purified, characterized, and tested for cytotoxic activity as follows. 14.5 ml of defatted ascites fluid was chromatographed on a Sephadryl-200 superfine column (Pharmacia Co.). The column, 26 mm in diameter and 850 mm long, was pre-equilibrated with 100 mM Tris-HCl buffer, pH 8.6. The same solution was used to elute the material at a flow rate of 1ml/min. The eluate was monitored at O.D.<sub>280</sub>, 15 and 4ml fractions collected. Figure 11 shows the elution profile. Based on SDS PAGE gel electrophoresis across the column using 20% gels, fractions 29-36 contained antibody. These fractions were pooled, diluted four fold with water, and chromatographed on a DEAE-Sephadex column (26mm x 130mm) previously equilibrated with 25mM Tris-HCl, pH 8.6. The column was rinsed once with 50ml 20 of 25 mM Na phosphate, pH7.0, and antibody eluted with a 500 ml gradient of Na phosphate, 25 to 200 mM, pH 7.0. The eluate was monitored at O.D.<sub>280</sub>, and 3ml fractions were collected. The elution profile is shown in Figure 12. The fractions were analyzed for antibody using SDS PAGE gel electrophoresis using 20% gels, and fractions 52-62 pooled, as were fractions 78-85 and 102-110, and shown to contain 25 520C9 antibody, bispecific antibody, and 3G8 antibody, respectively. The gel profiles are shown in Figure 13. The three fractions were sterile filtered, and tested for cytotoxic activity against chromium labeled SKBr3 cells as described above. The results are shown in Figure 14. It is apparent from the Figure that the 2B1 bispecific pool, at antibody concentrations ranging from 100ng/ml to 1ng/ml, displays a marked 30 increase in killing compared to the 520C9 or 3G8 pools.

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Having generally described what the applicant believes his invention to be, it will be appreciated by those skilled in the art that there are numerous materials and methods that can be substituted for those taught above, and that it is intended that these come within the scope of the invention. The invention is to be limited solely 5 by the scope of the appended claims.

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## WE CLAIM:

1. A method for selecting hybrid cells, comprising the steps of:
  - (a) Contacting a first cell type in a physiological solution containing a first fluorescent dye for a time and at an effective concentration that permits said first cell type to become labeled with an amount of said first fluorescent dye detectable by a fluorescent activated cell sorting machine, said first fluorescent dye having a negligible rate of efflux from said first cell type;
  - (b) Contacting a second cell type in a physiological solution containing a second fluorescent dye for a time and at an effective concentration that permits said second cell type to take up an amount of said second fluorescent dye detectable by a fluorescent activated cell sorting machine and an effective amount of a calcium channel blocker that maintains said second fluorescent dye at a detectable level inside said cell;
  - (c) Isolating said first and second cell types from said physiological solutions;
  - (d) Combining in a physiological solution said isolated first and second cell types in the presence of a fusogen to produce a mixture containing hybrid and non-hybrid cells; and
  - (e) Isolating hybrid cells comprising said first and second cell types from said solution mixture comprising passing said mixture through a fluorescent activated cell sorting machine.
2. The method as described in Claim 1, wherein said first cell type is a rodent cell.
- 25 3. The method as described in Claim 2, wherein said second cell type is a rodent cell.
4. The method as described in Claim 3, wherein said fluorescent dyes are selected from the group consisting of rhodamine 123 and hydroethidine.

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5. The method as described in Claim 3, wherein said first fluorescent dye is hydroethidine, and said second fluorescent dye is rhodamine 123.

6. The method as described in Claim 5, wherein said first and second cell types are mouse cells, and said first fluorescent dye is hydroethidine and said second 5 fluorescent dye is rhodamine 123.

7. The method as described in Claim 6, where: 1 said second cell type and said second fluorescent dye is in a physiological solution containing an effective amount of a calcium channel blocker that substantially prevents the efflux of rhodamine 123 from said mouse cells.

10 8. The method as described in Claim 7, wherein said calcium channel blocker is verapamil.

9. The method as described in Claim 8, wherein said verapamil is present at a concentration of about 10  $\mu$ g/ml.

10. The method as described in Claim 9, wherein said hydroethidine is 15 present in said physiological solution at a concentration of about 1-20 ug/ml.

11. The method as described in Claim 10, wherein said rhodamine 123 is present in said solution at a concentration of about 0.5-1.0 ug/ml.

12. The method as described in Claim 11, wherein said first and said second cell types are contacted with said hydroethidine or said rhodamine 123 for about 15- 20 45 minutes.

13. The method as described in Claim 11, wherein said first and second cell types are contacted with hydroethidine or rhodamine 123 at about 21-37°C for about 15-30 minutes.

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14. The method as described in Claim 7, wherein said mouse cells are hybridomas.
15. The method as described in Claim 1, wherein said solutions of steps (c), (d) and (e) contain an effective amount of a calcium channel blocker that maintains 5 said second fluorescent dye at a detectable level inside second cell type.
16. Hybrid cells produced by the method of Claim 1.
17. Hybrid cells produced by the method of Claim 7.
18. Hybrid cells produced by the method of Claim 13.
19. Hybrid cells produced by the method of Claim 13 selected from the 10 group consisting of hybridomas, triomas, and quadromas.
20. Antibody produced by the hybrid cells of claim 7.
22. Antibody produced by the hybrid cells of claim 13.
23. Antibody produced by the hybrid cells of claim 16.
24. Antibody produced by the hybrid cells of claim 19.
- 15 25. Hybridoma 2B1.
26. Antibody secreted by hybridoma 2B1.

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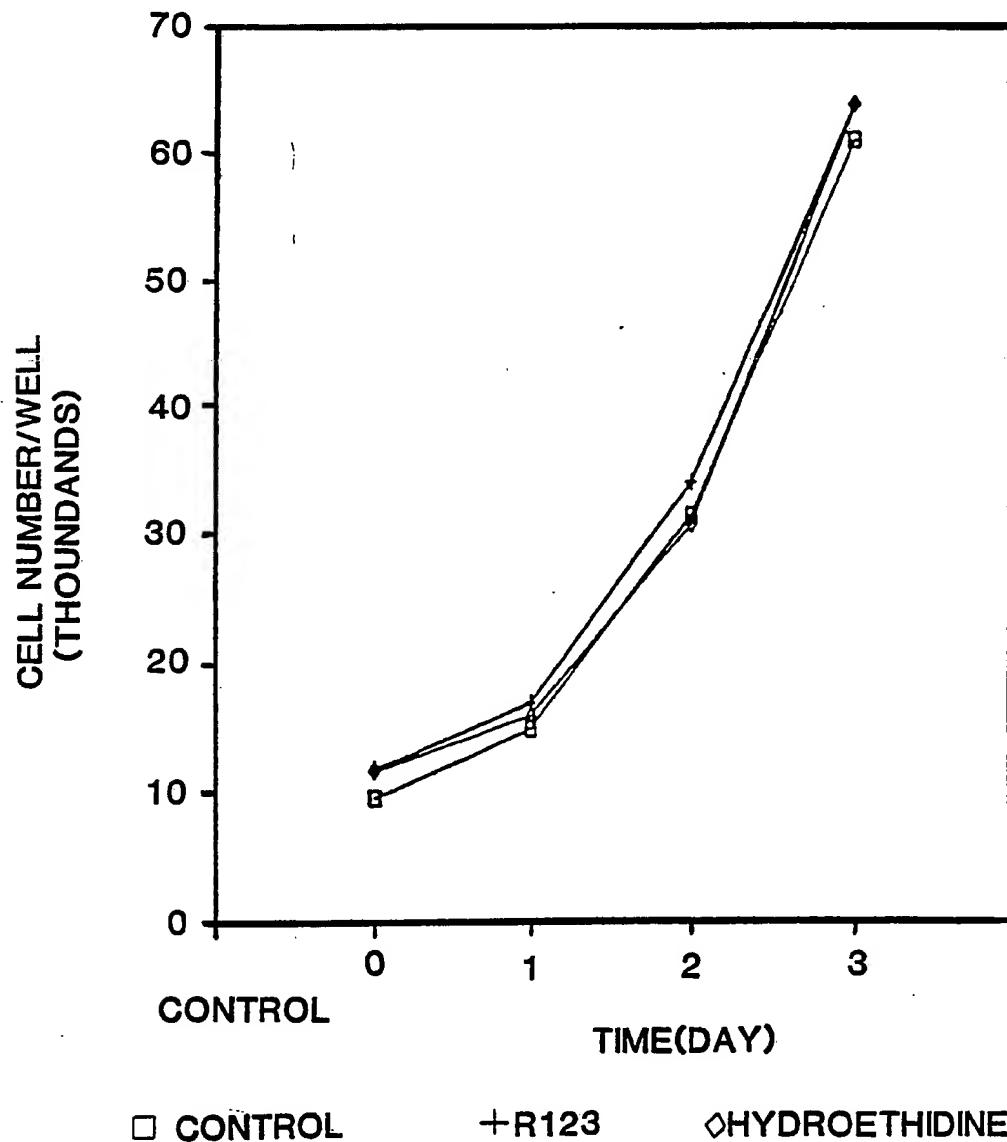


FIG. 1

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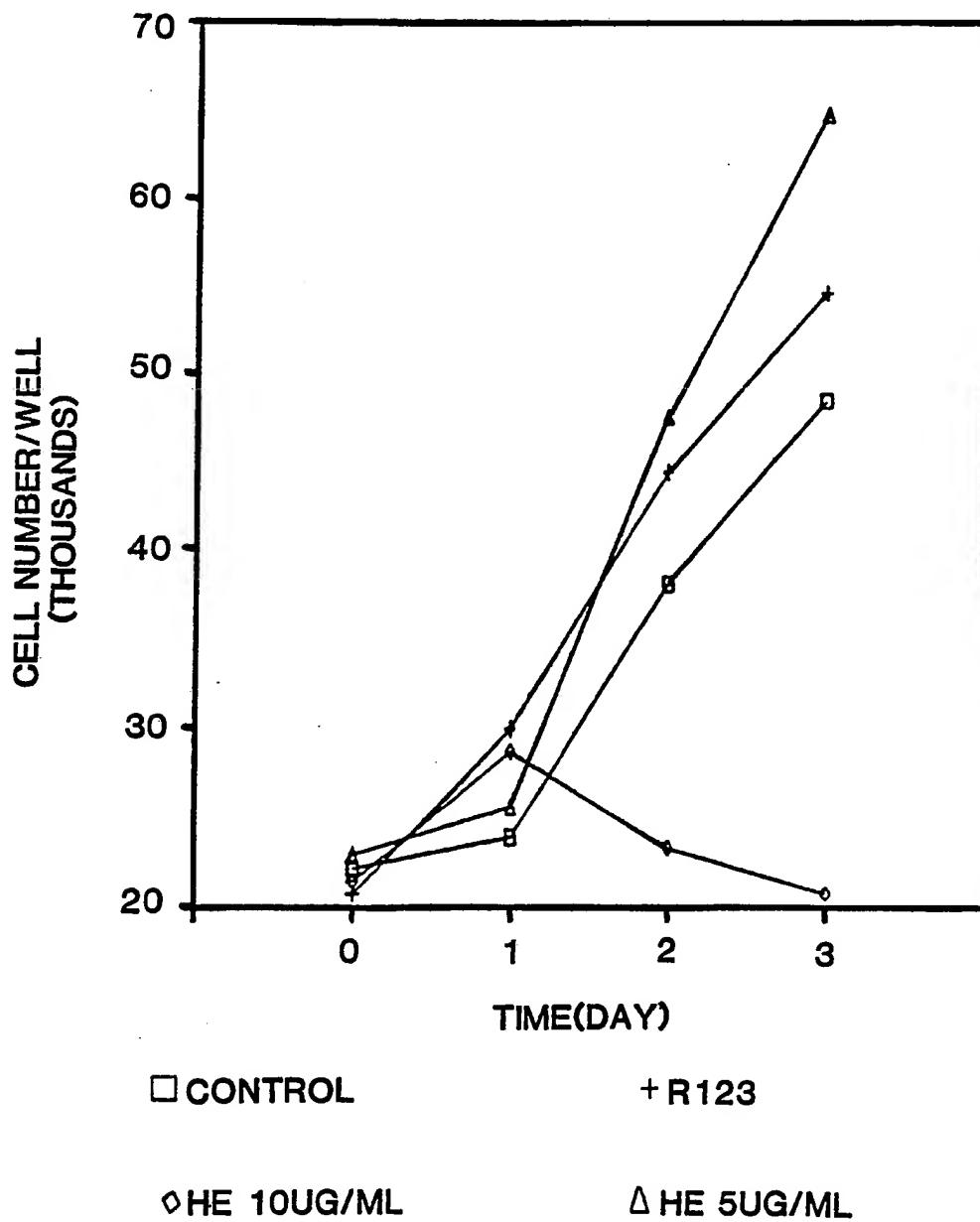


FIG. 2

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POSITION OF VERTICAL CURSOR  
X= 20  
HORIZONTAL CURSOR  
Y= 21  
LOWEST LEVEL

ADC5

1 2

3 4

DATA TO PRINTER

ADC4

1 2

3 4

AREA

1 2

3 4

RETURN TO CONTOUR

QUAD	PERCENT	PEAK POS	PEAK HT	AREA
1	0.01	0, 31	1	1
2	0.02	24, 27	1	3
3	12.08	0, 0	819	2416
4	87.9	31, 0	1829	17580

READY

**FIG. 3**

**FIG. 4**

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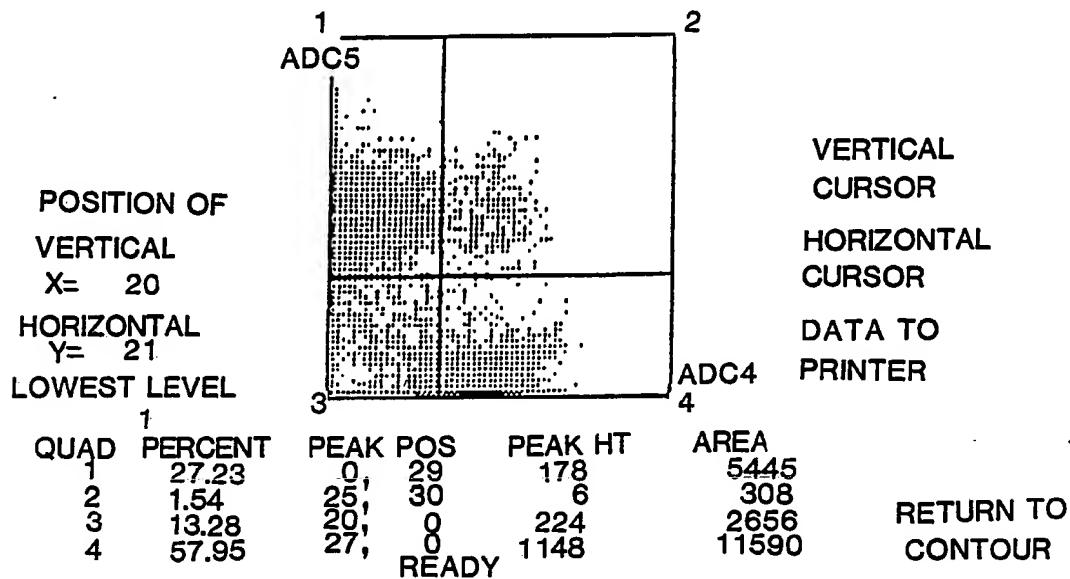
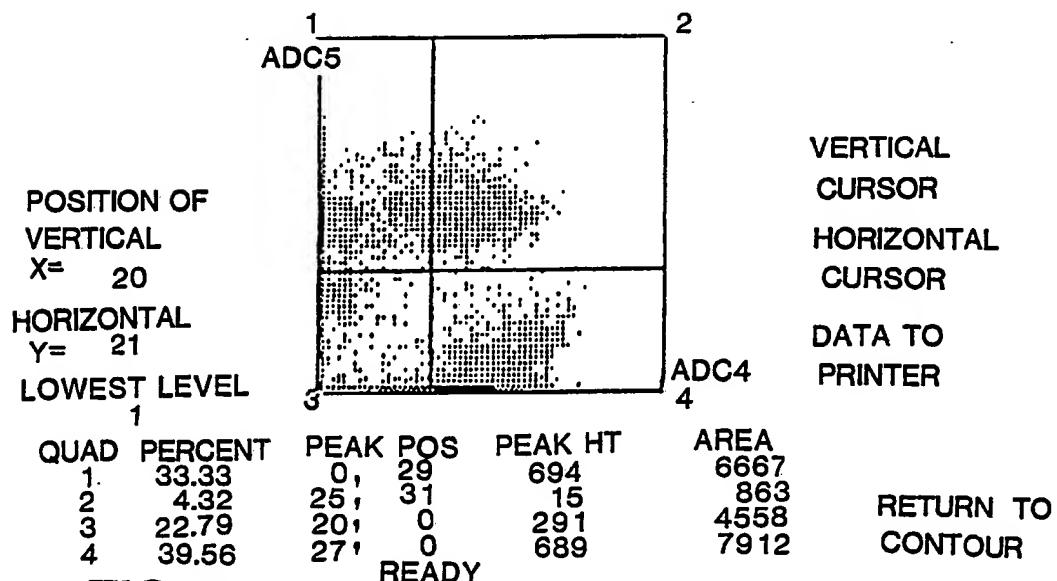


FIG. 5



**FIG. 6**

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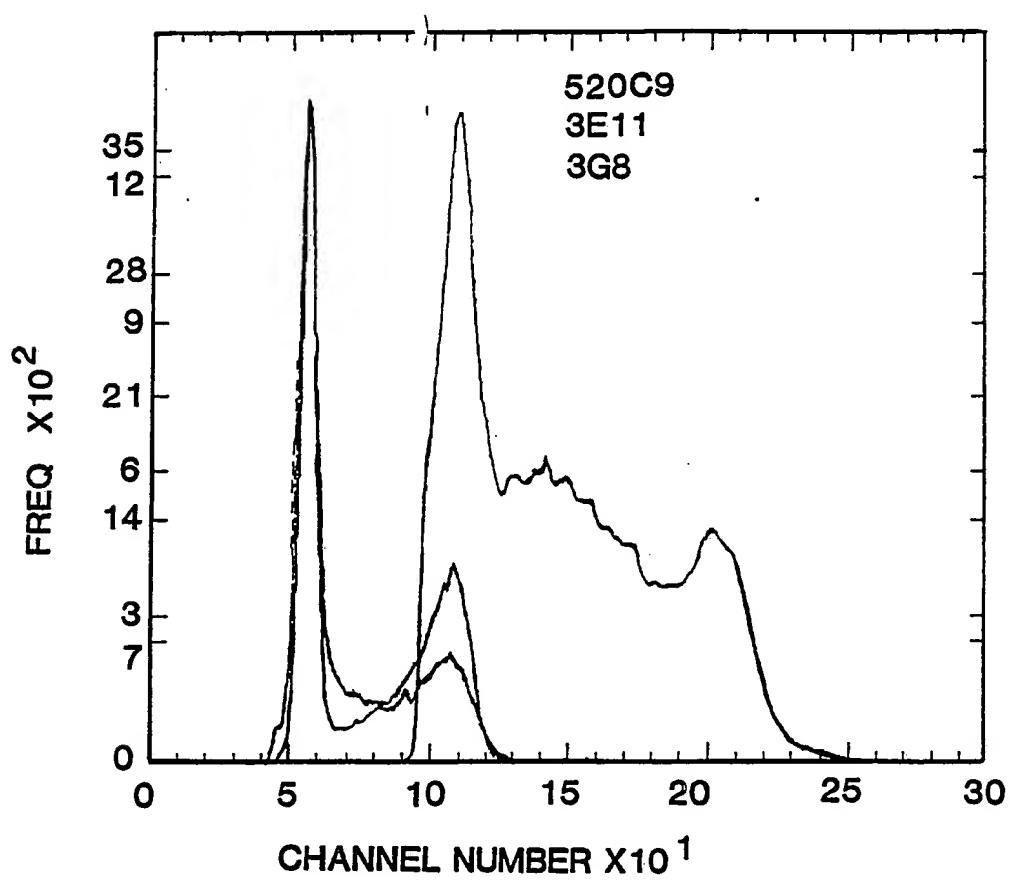


FIG. 7

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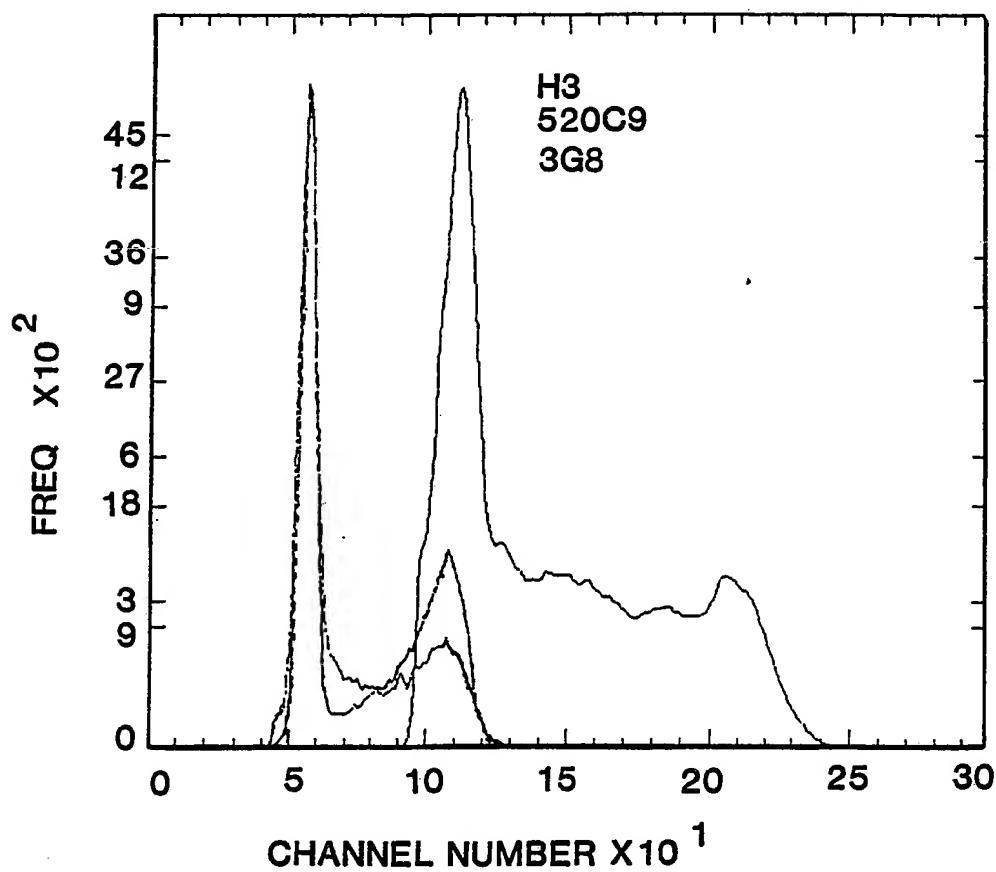


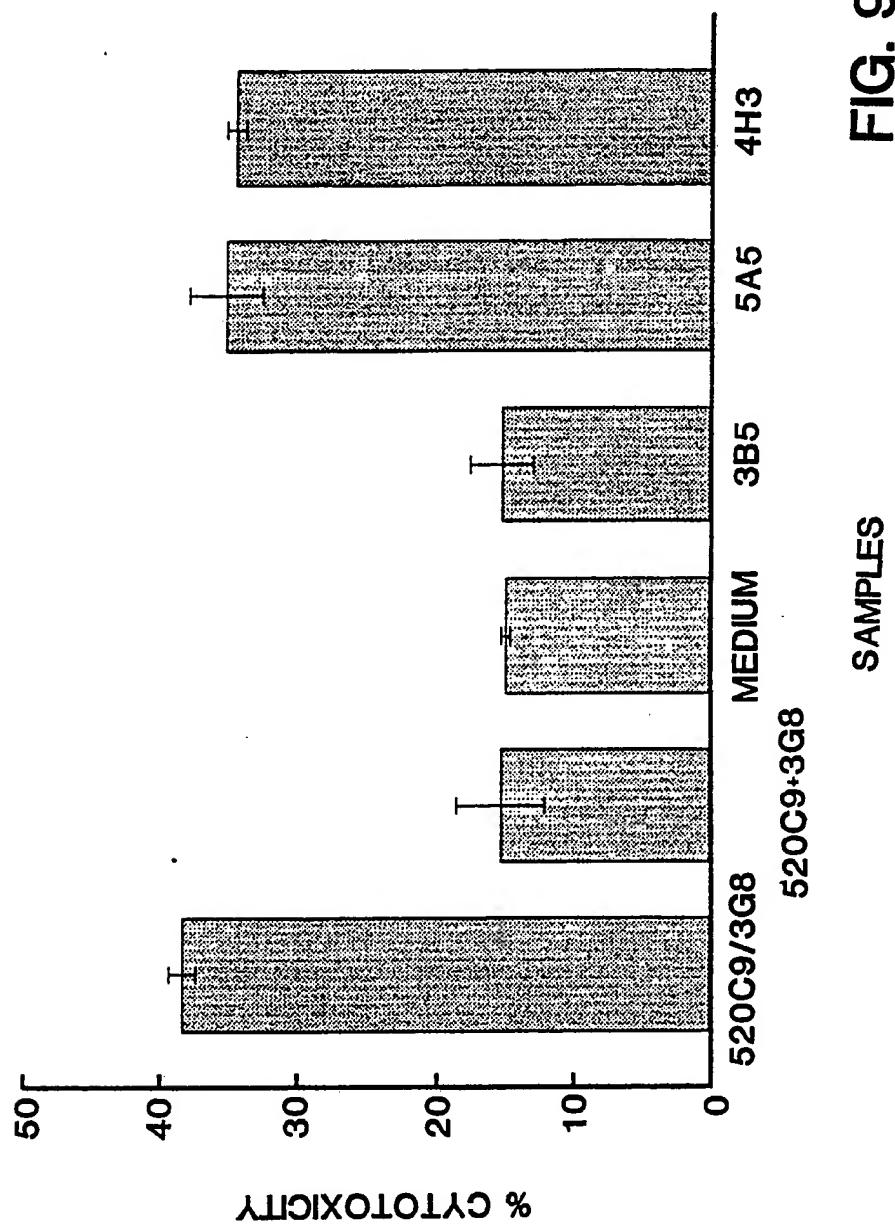
FIG. 8

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**FIG. 10**

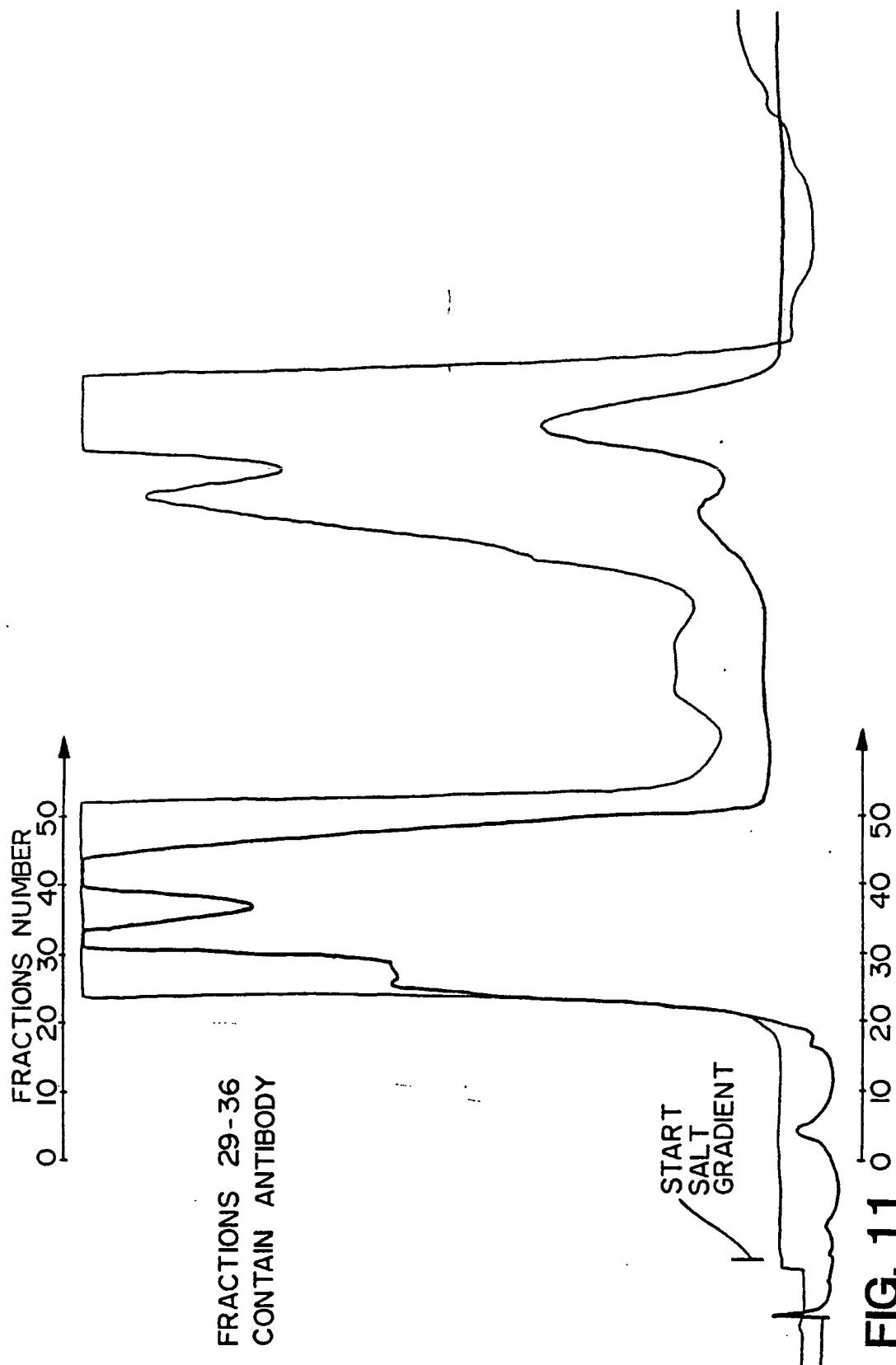
<u>Reagent</u>	<u>Concentration</u>	<u>% Specific Lysis</u>
<b>Donor #152</b>		
medium		29%
520C9-3G8 heteroconjugate	0.1 ug/ml	59%
520C9-3G8 + 520C9 + 3G8	0.1 ug/ml	78%
TS3 4H3	1/100	63%
TS1 5A5	1/100	67%
<b>Donor #154</b>		
medium		33%
520C9	1ug/ml	41%
520C9 + G8	1ug/ml	33%
520C9-3G8 + 520C9 + 3G8	0.1ug/ml	57%
TS3 4H3c3D7	1/500	56%
TS3 4H3c3E7	1/500	41%
TS1 5A5c2B1	1/500	45%
TS1 5A5c2D3	1/500	46%
negative control quadroma		
TS3 4H3c3D12	1/500	31%
<b>Donor #158</b>		
medium		20%
520C9	1ug/ml	37%
520C9-3G8 heteroconjugate	0.1ug/ml	60%
TS3 4H3c3D7	1/400	40%
TS1 5ASc2B1	1/400	45%

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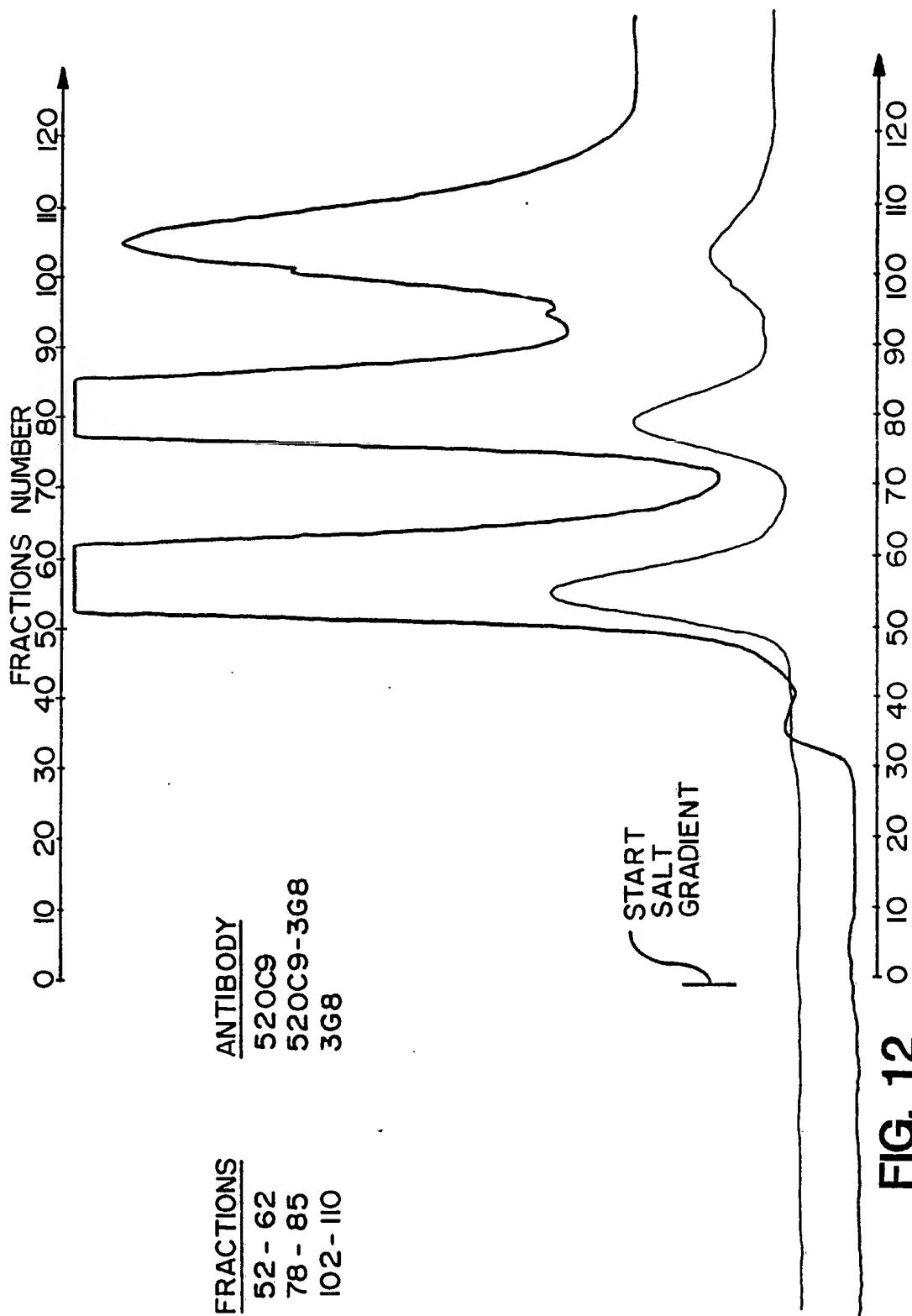


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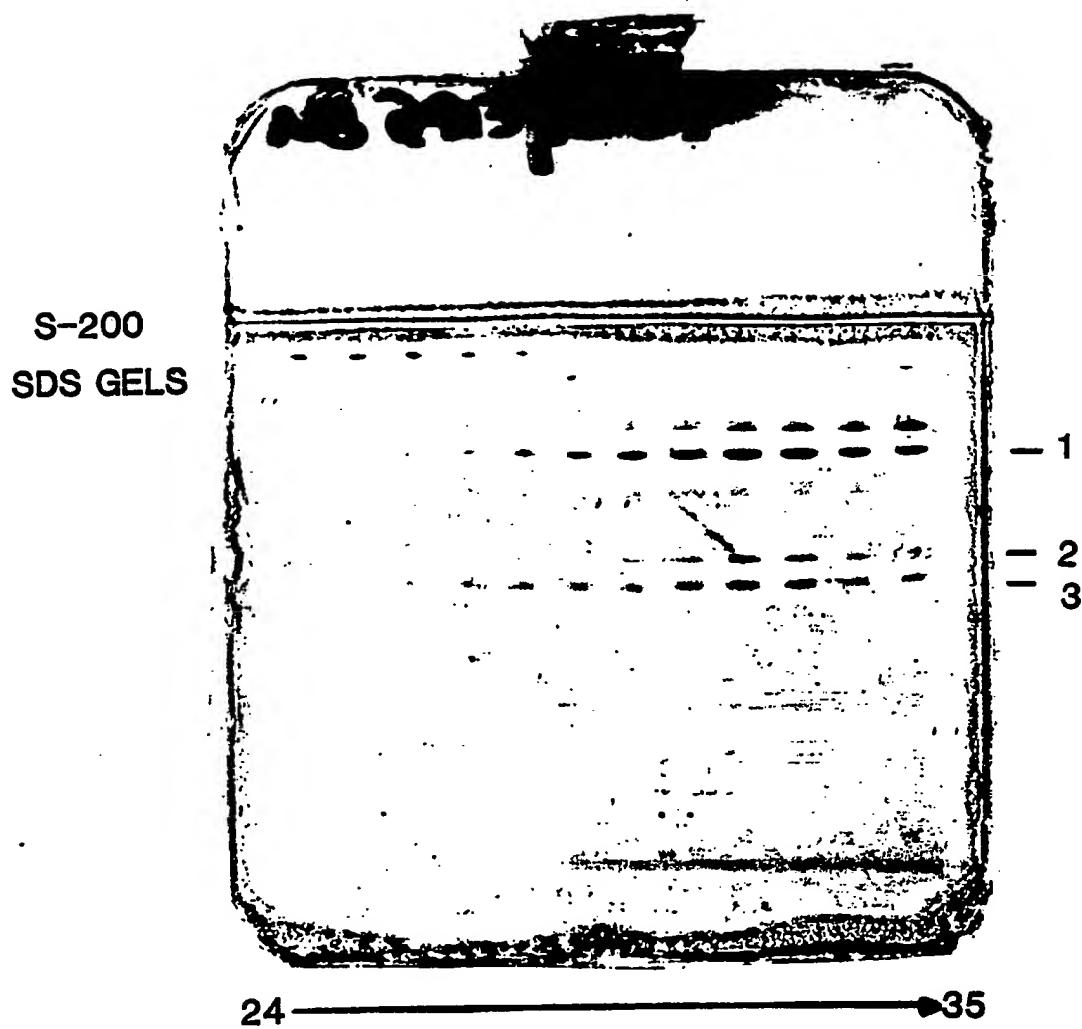


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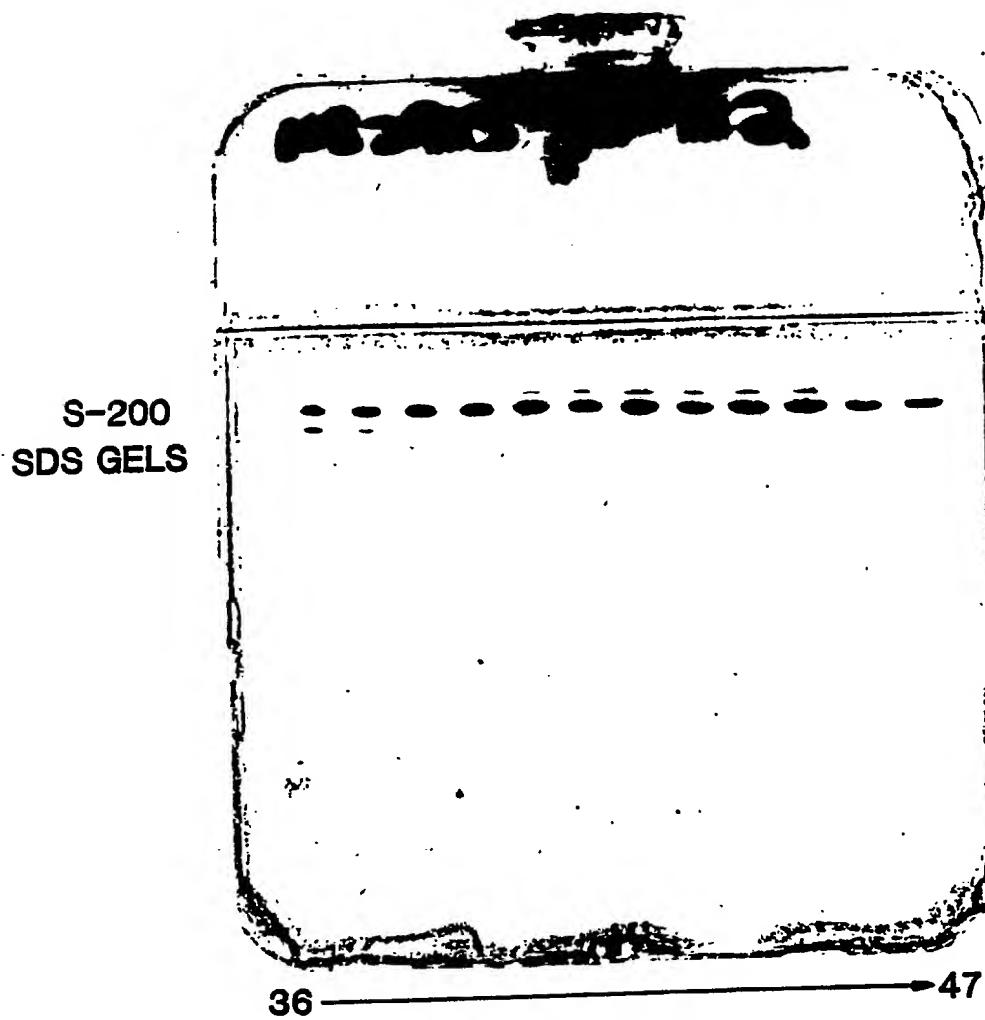
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**FIG. 13-1****COLUMN FRACTIONS****SUBSTITUTE SHEET**

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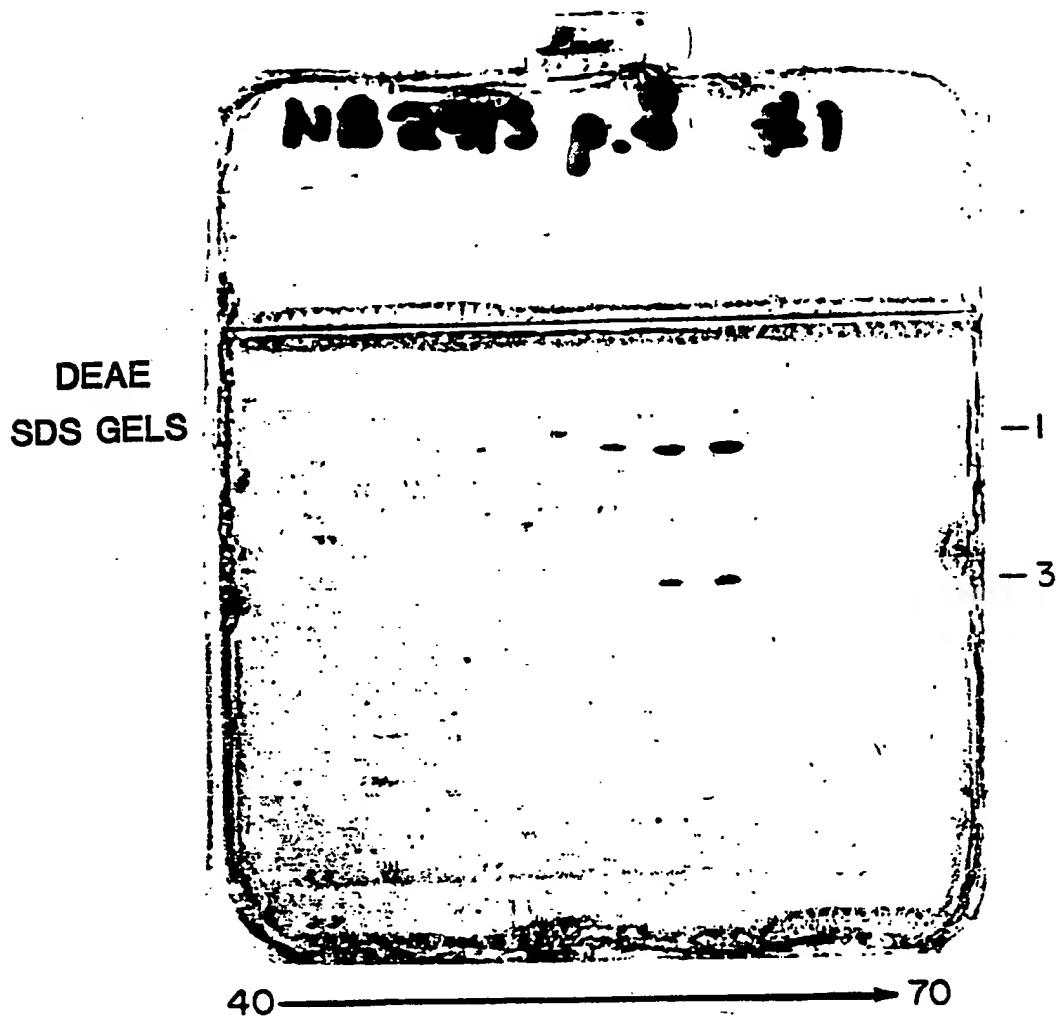
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**FIG. 13-2****COLUMN FRACTIONS****SUBSTITUTE SHEET**

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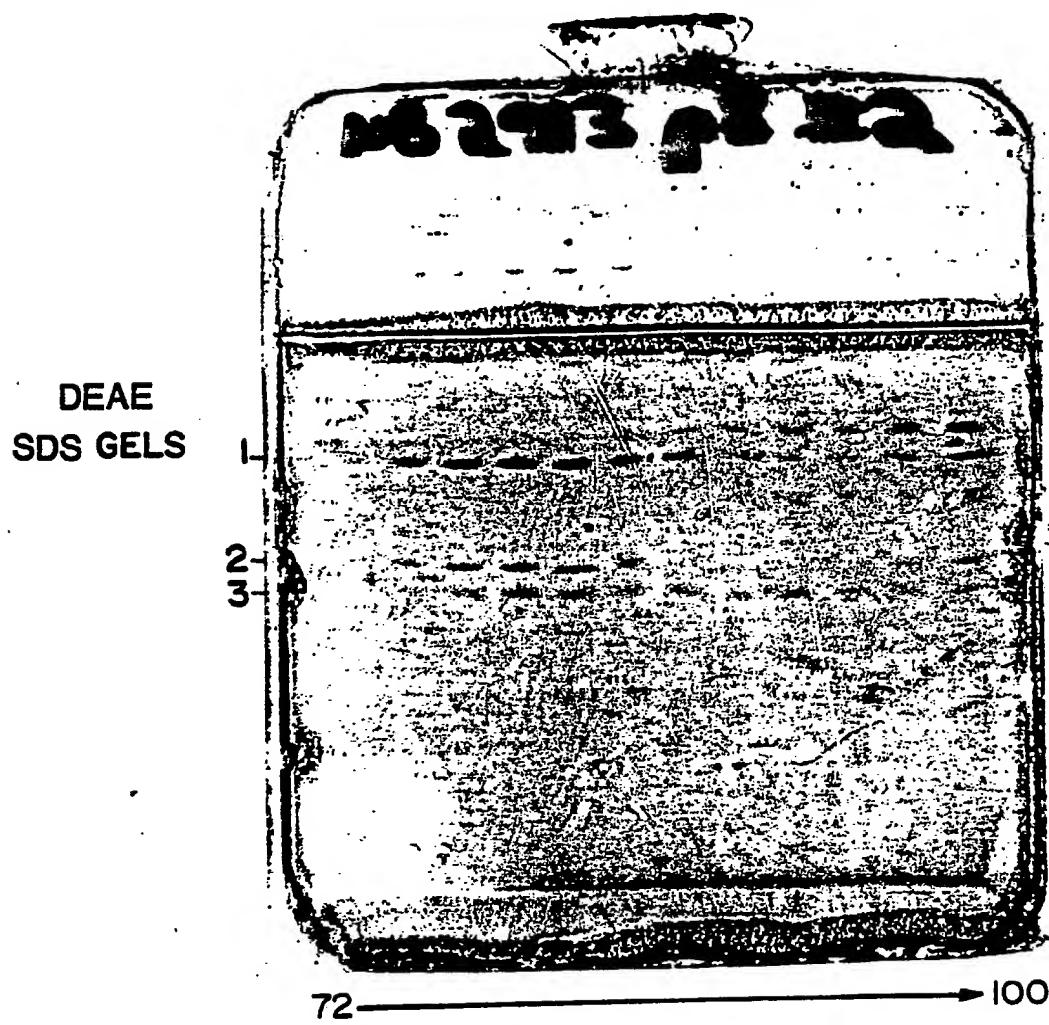
**FIG. 13-3****SUBSTITUTE SHEET**

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## FIG. 13-4



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**FIG. 14**

<u>Concentration</u>	<u>Antibody Fractions</u>		
	<u>520C9</u>	<u>520C9-3G8</u>	<u>3G8</u>
100ng/ml	13%	36%	16%
10ng/ml	18%	46%	13%
1 ng/ml	21%	52%	14%
0.1 ng/ml	10%	21%	18%
0.01 ng/ml	14%	19%	16%
0.001mg/ml	13%	20%	17%

<u>Concentration</u>	<u>520C9-3G8 Chemically Linked Heteroconjugate + 520C9 + 3G8 (Positive Control)</u>	<u>Unconjugated 520C9 + 3G8 (Negative Control)</u>
100ng/ml	40%	10%
10ng/ml	38%	10%
1 ng/ml	42%	9%

**SUBSTITUTE SHEET**

## INTERNATIONAL SEARCH REPORT

International Application No PCT/US 89/04023

## I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) \*

According to International Patent Classification (IPC) or to both National Classification and IPC  
 IPC<sup>5</sup> G 01 N 33/569, C 12 N 5/00, C 07 K 15/00, C 12 P 21/00, //  
 IPC: (C 12 P 21/00; C 12 R 1:91), // C 12 N 15/00

## II. FIELDS SEARCHED

Minimum Documentation Searched ?

Classification System	Classification Symbols
IPC <sup>5</sup>	C 12 N, G 01 N

Documentation Searched other than Minimum Documentation  
 to the Extent that such Documents are Included in the Fields Searched \*

## III. DOCUMENTS CONSIDERED TO BE RELEVANT\*

Category *	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	Journal of Immunological Methods, vol. 96, 1987, Elsevier Science Publishers B.V. (Biomedical Division), L. Karawajew et al.: "Bispecific antibody-producing hybrid hybridomas selected by a fluorescence activated cell sorter", pages 265-270, see the whole article --	1-6
X	Chemical Abstracts, vol. 108, no. 19, 9 May 1988 (Columbus, Ohio, US), see page 522, abstract no. 166012d, & DD, A, 249492 (AKADEMIE DER WISSENSCHAFTEN DER DDR) 9 September 1987 --	1-6
A	Chemical Abstracts, vol. 108, no. 3, 18 January 1988 (Columbus, Ohio, US), A. Krishan: "Effect of drug efflux blockers on vital staining of cellular DNA with Hoechst 33342", page 300, abstract no. 18663b, --	./.

\* Special categories of cited documents: <sup>10</sup>

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search

21st December 1989

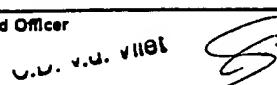
Date of Mailing of this International Search Report

06.02.90

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer



PCT/US 89/04023

International Application No. -2-

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	& Cytometry 1987, 8(6), 642-5 --	
A	EP, A, 0105614 (ORTHO DIAGNOSTIC SYSTEMS INC.) 18 April 1984 --	
A	US, A, 4499052 (M.J. FULWYLER) 12 February 1985 --	
A	EP, A, 0241042 (HITACHI LTD) 14 October 1987 -----	

**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.**

US 8904023  
SA 31267

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 26/01/90. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0105614	18-04-84	US-A- 4492752 AU-B- 557760 AU-A- 1864083 CA-A- 1202868 DE-A- 3377940 JP-A- 59065748	08-01-85 08-01-87 08-03-84 08-04-86 13-10-88 14-04-84
US-A- 4499052	12-02-85	DE-A, C 3331017 FR-A, B 2532431 GB-A, B 2126341 JP-A- 59060261 US-A- 4717655	08-03-84 02-03-84 21-03-84 06-04-84 05-01-88
EP-A- 0241042	14-10-87	JP-A- 63045563	26-02-88